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General Approach for Characterizing In Vitro Selected Peptides with Protein Binding Affinity

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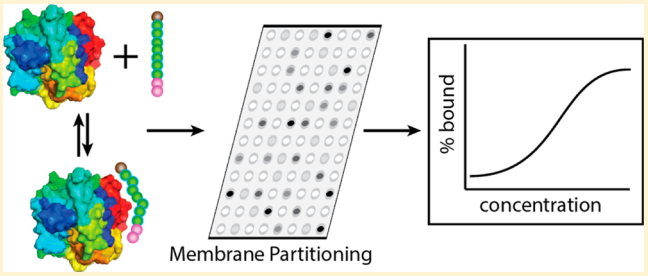
Supporting Information

ABSTRACT: In vitro selection technologies are important tools for identifying high affinity peptides to proteins of broad medical and biological interest. However, the technological advances that have made it possible to generate long lists of candidate peptides have far outpaced our ability to characterize the binding properties of individual peptides. Here, we describe a low cost strategy to rapidly synthesize, purify, screen, and characterize peptides for high binding affinity. Peptides are assayed in a 96-well dot blot apparatus using membranes that enable partitioning of bound and unbound peptide−protein complexes. We have validated the binding affinity constants produced by this method using known peptide ligands and applied this process to discover five new peptides with nanomolar affinity to human α-thrombin. Given the need for new analytical tools that can accelerate peptide discovery and characterization, we feel that this approach would be useful to a wide range of technologies that utilize high affinity peptides.



In vitro selection technologies have become indispensible tools for identifying high affinity peptides to proteins of broad medical and biological interest.[1] However, the technological advances that have made it possible to generate long lists of candidate peptides have far outpaced our ability to characterize the binding properties of individual peptides. This disparity is due, in part, to recent advances in DNA sequencing technology, which have made it possible to generate millions of peptide sequences from a single in vitro selection experiment.[2] Other factors that have contributed to the rise in peptide sequence discovery include the use of bar coded libraries and liquid handling robots in selection protocols.[3]

Countering these advances is the slow pace at which individual peptides are characterized. In many cases, peptides identified by in vitro selection are produced by solid-phase synthesis, purified by HPLC chromatography, and assayed for function using analytical techniques, such as surface plasmon resonance (SPR), that require large amounts of highly pure peptide or protein. Because this process is both time-consuming and costly, many researchers have turned to column binding assays as a way to quickly screen in vitro selected peptides for high affinity binding.[4] Although such assays are relatively easy to perform and use only small amounts of peptide, the data produced is not quantitative. These assays also suffer from high background and problems caused by differential peptide expression, which can make it difficult to compare different peptides analyzed in side-by-side assays.[4b] Even when high affinity peptides are discovered, additional experiments are needed to obtain quantitative metrics, such as equilibrium binding affinity constants (K_d) that help describe the physical properties of the peptide−protein interaction.

Recognizing the limitations of traditional methods, we sought to develop a new analytical technique to identify high affinity peptides from enriched pools of in vitro selected sequences. Our goal was to develop a rapid and inexpensive method that would make it possible to rank selected peptides based on their relative binding affinity and, in a second step, determine the K_d value for the subset of high affinity ligands. The challenge was to design a system that would require minimal amounts of peptide and protein, was amenable to diverse protein classes, and allowed individual assays to be performed in a parallel format.

We envisioned an overall system in which peptides generated by cell-free expression would be brought to equilibrium with their cognate protein, and bound peptide−protein complexes would be separated from the unbound peptide using a double-filter binding assay (Figure 1). We felt that this strategy has a number of key advantages over existing methods. First, cell-free peptide synthesis makes it possible to synthesize large numbers of different peptide sequences in a fraction of the time that it would take to obtain the same constructs by solid-phase synthesis (hours vs days).[5] Second, peptides made by cell-free synthesis can be engineered to carry a protein affinity tag, which allows for purification by affinity chromatography. Third,

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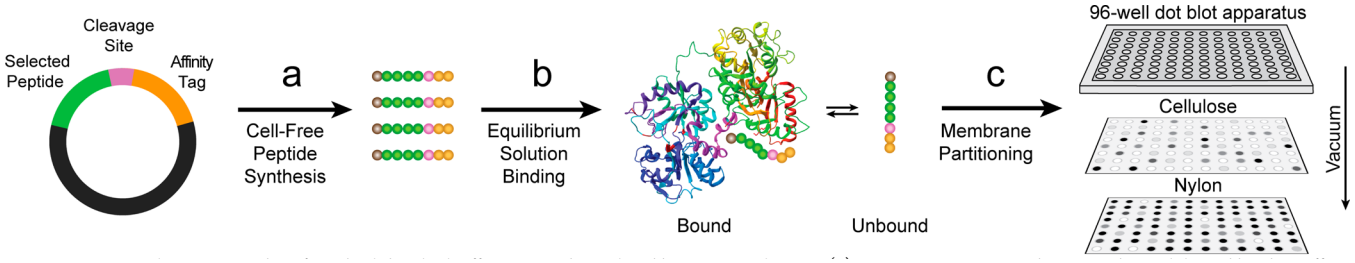

Figure 1. General strategy to identify and validate high affinity peptides isolated by in vitro selection. (a) DNA sequences encoding peptides with ligand binding affinity are inserted into a custom peptide expression vector, expressed in a coupled cell-free transcription–translation system as ^{35}S-labeled peptides and purified by affinity chromatography. (b) Peptides are equilibrated in solution with their cognate protein target. (c) Bound and unbound peptides are separated in a 96-well dot blot apparatus by passing the mixture through a two-membrane system.

peptides produced by cell-free synthesis can be labeled with ^{35}S-methionine, a radioisotope that allows for accurate detection at low concentrations without altering the physical properties of the peptide. Fourth, filter-binding assays provide a useful method for determining K_d values, as binding can be measured across a range of protein concentrations.[6] Last, the entire process can be performed in parallel, which makes it possible to simultaneously analyze the binding properties of many different peptides.

To test this strategy, we designed a custom peptide expression vector that would encode our peptide of interest followed by a TEV protease cleavage site and the amino acid sequence for the streptavidin binding peptide (SBP).[7,8] We anticipated that the SBP tag would provide a convenient positive control for subsequent binding assays, since the SBP–streptavidin interaction is well-known.[9] In our peptide expression assays, we found that peptides generated in rabbit reticulocyte lysate could be purified from the crude lysate by affinity capture on streptavidin-coated agarose beads. We developed two elution strategies to recover the peptide from the beads. The first strategy involved eluting the beads with deionized water, which allowed us to obtain the peptide of interest as an SBP fusion peptide for control assays with streptavidin. The second strategy involved eluting the peptide of interest as a free peptide by incubating the beads with TEV protease, which separated the peptide of interest from the SBP portion of the fusion.

While the synthesis and purification of SBP-tagged peptides proceeded without problem, developing a two-membrane system that could efficiently partition bound peptide–protein complexes from the unbound peptide proved more challenging. Although filter-binding assays represent an established method for studying the binding properties of protein–DNA interactions[7,10] and more recently have been extended to include protein–XNA complexes,[11] such systems have not been developed for protein–peptide interactions. This is presumably due to the fact that nitrocellulose has a general nonspecific affinity for amino acids, which precludes its ability to distinguish peptides from proteins.

To identify a suitable membrane pair, we evaluated the binding properties of several common laboratory membranes with different surface compositions and pore sizes in a dot blot apparatus. We tested nitrocellulose, PVDF, nylon, and cellulose membranes with various pore sizes. However, reproducible results were only observed using a double filter membrane setup with a top layer composed of regenerated cellulose and a bottom layer composed of nylon. Using this membrane configuration, peptide–protein complexes were retained on the top cellulose membrane and unbound peptides that passed through the top membrane were captured on the lower nylon membrane.

We tested the reproducibility of the cellulose–nylon membrane system by performing a 96-well dot-blot assay using SBP and streptavidin to represent a model peptide–protein complex. In this binding assay, SBP-tagged peptides labeled with ^{35}S-methionine were equilibrated in phosphate buffered saline (PBS, pH 7) solutions that either lack or contain streptavidin (50 nM). After 1 h of incubation at 25 °C, the solutions were loaded into the dot blot apparatus and passed through cellulose and nylon membranes. The membranes were removed, dried, and quantified by phosphorimaging (Figure 2a). Analysis of the individual spots allowed us to quantify the

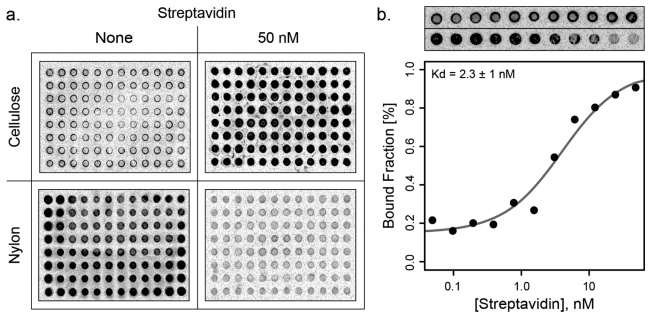

Figure 2. Two-membrane double-filtration system for separating bound and unbound peptide–protein complexes. (a) Analysis of the streptavidin binding peptide (SBP) on cellulose and nylon membranes in the absence and presence of streptavidin protein. (b) Equilibrium dissociation plot measuring the binding interaction of SBP with streptavidin.

amount of SBP peptide present on the cellulose and nylon membranes. We found that 89 ± 2% of the ^{35}S-labeled peptide was retained on the cellulose membrane when streptavidin was present in the PBS buffer. By contrast, only 19 ± 6% of the ^{35}S-labeled peptide remained on the cellulose membrane when streptavidin is absent from the buffer. While some variability was observed across the members, this result suggested to us that the cellulose–nylon double-filter system should be sufficient to distinguish the binding properties of different peptides.

For the cellulose–nylon system to function as an accurate predictor of peptide binding affinity, it was necessary to confirm that equilibrium was maintained during the filtration step. If equilibrium were disrupted as the peptide–protein complexes passed through the membranes, then the observed binding values would underestimate the true binding affinity of the

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peptide. To explore this possibility, we measured the binding affinity constant of the SBP−streptavidin interaction using the double-filter assay. SBP-tagged peptides labeled with ^{35}S-methionine were equilibrated for 1 h in PBS solutions that contained a range of streptavidin protein concentrations. The solutions were loaded into the dot blot apparatus and passed through the cellulose−nylon membranes (Figure 2b). Analysis of the bound fraction at each SBP concentration yielded a binding isotherm with a K_d of 2.3 ± 1 nM, which is consistent with the literature value of 2.4 nM.[8] On the basis of this result, we concluded that equilibrium is maintained for high affinity peptide−protein complexes.

One of the problems facing those that attempt to identify high affinity peptides to proteins of medical or biological interest is the challenge of distinguishing the highest affinity peptides from a list of in vitro selected peptide sequences. In many cases, the highest affinity peptides are not the most abundant sequences or even the sequences that share a common motif.[2] Recognizing this problem, we wondered if our cellulose−nylon membrane system could be used to identify and characterize high affinity peptides from a set of in vitro selected sequences. To explore this possibility, we identified an mRNA display selection in which a random library of 10^{11} different mRNA−peptide fusions was used to isolate peptides that could bind to human α-thrombin.[12] The authors reported 45 sequences that remained in the pool after 10 successive rounds of in vitro selection and amplification. Using a column binding assay, two peptides (T10.39 and T10.11) were identified with high affinity to the protein target. Both peptides were synthesized and tested for binding by surface plasmon resonance. T10.39 was found to bind human α-thrombin with a K_d of 166 nM, while T10.11 bound with a K_d of 520 nM.[12]

Considering the possibility that some high affinity peptides may have been overlooked due to the limitations of the original column binding assay, we decided to test 24 of the 45 sequences in our cellulose-nylon membrane system. The set of 24 peptides were randomly chosen (Figure 3a), inserted into our custom peptide expression vector, expressed in rabbit reticulocyte lysate as ^{35}S-labeled peptide fusions, purified on streptavidin coated agarose beads, and eluted by TEV protease cleavage of the fusion peptide. Three of the peptides (T10.35, T10.46, and T10.57) did not express well and were discarded. Coincidentally, these three peptides also have high hydrophic values, indicating that our screen could be an indicator of peptide solubility. The remaining peptides were each separately incubated with 250 and 500 nM human α-thrombin for 1 h at room temperature and analyzed in parallel by passing the solutions through the cellulose−nylon membrane system. Control samples lacking thrombin were used to define the level of background binding to the membrane and the fraction of bound peptide was compared to T10.39, a high affinity thrombin-binding peptide (Figure 3b).

From our filter-binding assay, we discovered five previously uncharacterized sequences (T10.06, T10.13, T10.25, T10.30, and T10.37) that exhibit at least 25% binding to human α-thrombin when the protein was poised at a concentration of 250 nM. The remaining sequences showed little or no binding, indicating that these sequences are all weak affinity ligands. Of the high affinity peptides, three contain a conserved DPGR motif that is found in T10.39, while the other two show no similarity to T10.39 or each other. This could suggest the peptides bind different sites on the surface of human α-thrombin, with the DPGR containing peptides targeting the

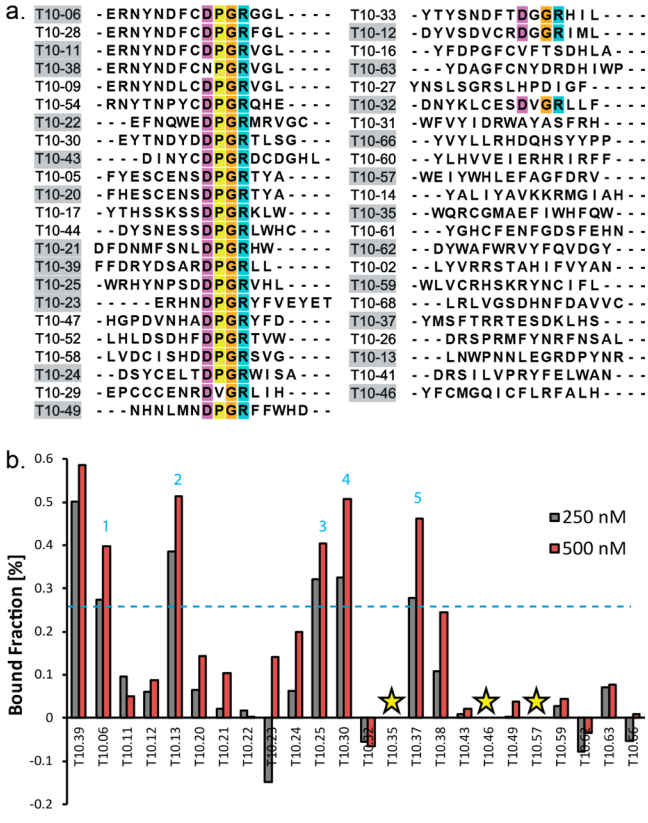

Figure 3. Screen of in vitro selected thrombin-binding peptides. (a) A list of 45 thrombin-binding peptides. Peptides selected for dot blot analysis (gray).[12] (b) Membrane-based screen of 24 thrombin-binding peptides for affinity to human α-thrombin. Stars indicate peptides with expression levels below the detection limit for dot blot analysis. High affinity peptides are numbered in blue. Arbitrary threshold (dashed blue line).

same epitope as T10.39 and the two unique peptides binding elsewhere on the surface; however, further experiments are needed to test this hypothesis.

Given the importance of human α-thrombin as a potential therapeutic target,[13] we sought to determine the binding affinity of the five novel thrombin-binding peptides. We began by validating our filter-binding assay using peptide T10.39, which was previously characterized and found to bind thrombin with a K_d of 166 nM.[12] Peptide T10.39 was expressed and purified as described above for the SBP peptide. The peptide was then incubated with a range of thrombin concentrations, and peptide−protein complexes were separated from the free peptide by passing the solutions through the cellulose−nylon membranes. Analysis of the binding isotherm revealed K_d of 170 ± 40 nM, which closely approximates the known literature value (Figure 4a).[12] Moreover, no difference was observed when the T10.39 was measured as a free peptide or as an SBP fusion peptide (data not shown).

Next, we measured the equilibrium binding affinity of the five uncharacterized peptides using the same methodology described above. In each case, the peptides were expressed and purified as ^{35}S-labeled free peptide by eluting the peptides from the beads with TEV protease. The five peptides were incubated with a range of thrombin concentrations and their K_d values were determined by quantifying the amount of ^{35}S-label on the cellulose and nylon membranes. Peptides T10.25, T10.30, and T10.6, which share the DPGR motif with T10.39,

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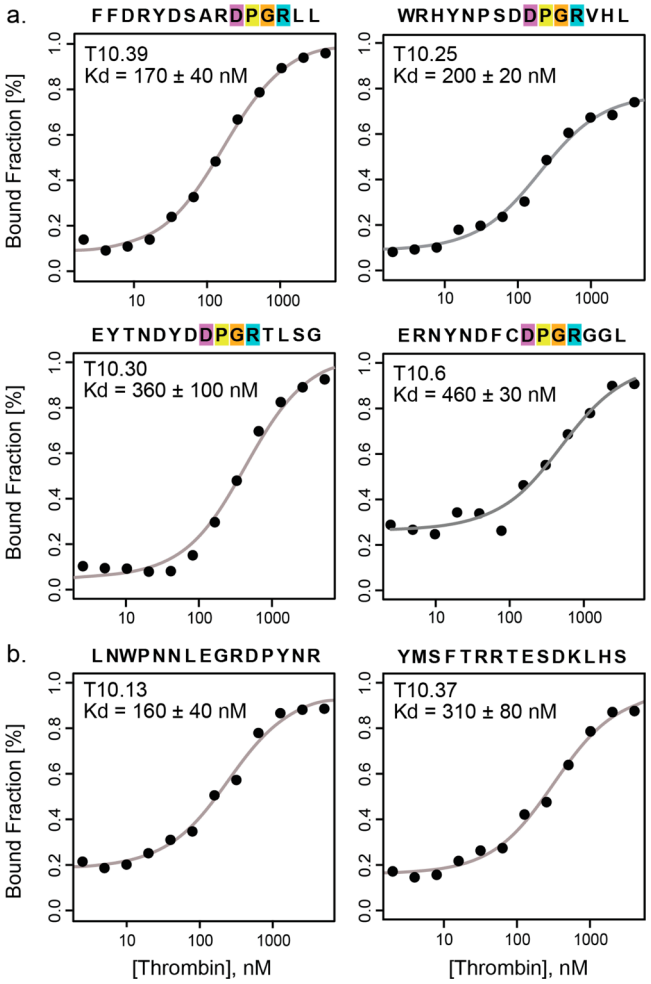

Figure 4. Equilibrium dissociation plots measuring the binding affinity for six high affinity thrombin-binding peptides. Binding isotherms were compared for peptides containing (a) and lacking (b) a conserved DPGR motif.

have K_d values of 200, 360, and 460 nM, respectively (Figure 4a). By contrast, peptides T10.13 and T10.37, which are unique with respect to the T10.39 sequence, have K_d values of 160 and 310 nM, respectively (Figure 4b).

To further validate our results, we produced peptides T10.13 and T10.37 by solid-phase synthesis, purified both sequences by HPLC chromatography, and confirmed their binding affinity by microscale thermophoresis (Figure S2 in the Supporting Information). This technique measures changes in the hydration shell (due to conformational changes) along a temperature gradient, which makes it possible to determine K_d values using minimal amounts of sample.[14] We found that peptides T10.13 and T10.37 bind thrombin with K_d values of 180 and 290 nM, which closely approximates the K_d values obtained using our double-filter binding assay.

In addition, we also demonstrated that the high affinity peptides function in a complex biological medium. In this case, peptides T10.13 and T10.37 were conjugated to streptavidin-coated magnetic beads and used to recover recombinant human α-thrombin that had been doped into HeLa cell lysate. After an incubation of 1 h at 25 °C, the beads were precipitated, the supernatant was removed, and the beads were washed with TBST buffer. The supernatant and bead samples were analyzed by SDS acrylamide gel electrophoresis. Both peptides pulled-down human α-thrombin from the cell lysate with efficiencies similar to peptide T10.39 and no contamination above the bead-only control was observed (Figure S3 in the Supporting Information), demonstrating high affinity and high specificity binding.

The past few years have witnessed an explosion in the demand for high-quality peptides that can be used to support a growing industry of peptide-based therapeutic and diagnostic applications. Unlike antibodies, peptides are amenable to chemical synthesis, generally nonimmunogenic, and their small size allows them to penetrate further into soft tissue.[15,16] These properties, along with improved strategies for increasing serum stability, warrant new methods to streamline the peptide discovery process.[17] In line with these efforts, we present a general approach for characterizing the binding properties of in vitro selected peptides. This approach provides an inexpensive method to synthesize, purify, screen, and characterize peptides for high-affinity binding to their cognate protein target. We validated the method using peptides with known protein-binding interactions and applied the strategy to identify five new peptides that bind to human α-thrombin with nanomolar affinity and high specificity.

In summary, we provide a new analytic technique to rapidly screen and characterize in vitro selected peptides with high protein binding affinity. We have successfully evaluated peptides that range in size from 22 to 74 amino acids and exhibit binding affinity constants of 1−500 nM. While it is likely that subnanomolar binding affinities could be measured using this approach, we suspect that weaker interactions may not be possible due to long transit times through the membrane. During the course of our study, we noticed that peptides that do not express well by in vitro translation tend to have high hydrophobic values, suggesting that peptide recovery after expression and purification could be an indicator of peptide solubility. This observation could provide a simple way to determine whether a peptide will be soluble in an aqueous solution. Relative to more conventional analytical techniques, like SPR or isothermal titration calorimetry (ITC), the method presented here allows for rapid screening of multiple peptide candidates in small sample volumes using cell-free translated peptides that can be obtained in a cost-effective manner. By contrast, SPR and ITC generally require large amounts of purified peptide and/or protein that can be cost prohibitive when screening large numbers of peptides. While our approach is ideal for peptide screening and characterization, high affinity ligands discovered using this method may require further characterization in order to obtain a complete kinetic and thermodynamic profile of the peptide−protein interaction. Recognizing the advantages of small sample volumes, low cost, and high throughput, we suggest that this strategy could be used to accelerate the pace of peptide characterization and help advance the development of peptide-based affinity reagents.[18]

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, MST analysis, and pull-down data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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